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ORIGINAL ARTICLE

Group I metabotropic glutamate receptors in the primate motor thalamus: subsynaptic association with cortical and sub-cortical glutamatergic afferents

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Abstract
Preclinical evidence indicates that mGluR5 is a potential therapeutic target for Parkinson's disease and L-DOPA-induced dyskinesia. However, the mechanisms through which these therapeutic benefits are mediated remain poorly understood. Although the regulatory role of mGluR5 on glutamatergic transmission has been examined in various basal ganglia nuclei, very little is known about the localization and function of mGluR5 in the ventral motor and intralaminar thalamic nuclei, the main targets of basal ganglia output in mammals. Thus, we used immuno-electron microscopy to map the cellular and subcellular localization of group I mGluRs (mGluR1a and mGluR5) in the ventral motor and caudal intralaminar thalamic nuclei in rhesus monkeys. Furthermore, using double immuno-electron microscopy, we examined the subsynaptic localization of mGluR5 in relation to cortical and sub-cortical glutamatergic afferents. Four major conclusions can be drawn from these data. First, mGluR1a and mGluR5 are expressed postsynaptically on the plasma membrane of dendrites of projection neurons and GABAergic interneurons in the basal ganglia- and cerebellar-receiving regions of the ventral motor thalamus and in CM. Second, the plasma membrane-bound mGluR5 immunoreactivity is preferentially expressed perisynaptically at the edges of cortical and sub-cortical glutamatergic afferents. Third, the mGluR5 immunoreactivity is more strongly expressed in the lateral than the medial tiers of CM, suggesting a preferential association with thalamocortical over thalamostriatal neurons in the primate CM. Overall, mGluR5 is located to subserve powerful modulatory role of cortical and subcortical glutamatergic transmission in the primate ventral motor thalamus and CM.

Keywords Globus pallidus · Cerebellum · Rhesus monkey · mGluR · Parkinson's disease

Introduction

Glutamate is the main excitatory neurotransmitter in the CNS that elicits its action through the activation of ionotropic (iGlu) and metabotropic glutamate receptors (mGluRs). iGlu receptors, including N-Methyl-d-Aspartate (NMDA), α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors are ligand-gated ion channels that promote rapid excitatory neurotransmission, while mGluRs are a family of G protein-coupled receptors that "modulate" glutamatergic and non-glutamatergic transmission through pre- and post-synaptic mechanisms (Conn and Pin 1997; Niswender and Conn 2010; Nakanishi 1994). Because of their modulatory role, mGluRs have become a major target of drugs for various brain diseases, some of which (Parkinson disease, Huntington's disease, Schizophrenia, Alzheimer's disease, Fragile-X syndrome) being tightly linked with thalamocortical dysfunction (Conn et al. 2005; Ossowska et al. 2007; Gasparini et al. 2008; Niswender and Conn 2010; Nicoletti et al. 2011; Vaidya et al. 2013; Johnson et al. 2009). However, despite their abundance and widespread expression through the mammalian thalamus, the functional roles of thalamic mGluRs remains poorly understood (Govindaiah et al. 2012b; Pressler and Regehr 2013; Sherman 2014; Salt and Eaton 1996).

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The mGluRs family comprises eight receptor subtypes classified into three groups on the basis of their amino acid sequence homology, signal transduction mechanisms and pharmacological profiles. Group I receptors (mGluR1 and 5) are linked to the activation of phospholipase C and generally mediate postsynaptic excitatory effects, whereas group II (mGluR2 and 3) and group III (mGluR4, -6, -7, and -8) receptors are negatively coupled to adenylyl cyclase and generally mediate presynaptic inhibitory influences on neurotransmitter release (Conn and Pin 1997; Nakanishi 1994). Extensive preclinical studies have confirmed the therapeutic relevance of mGluR5 allosteric modulators for the treatment of a number of psychiatric disorders, epilepsy and neurodegenerative diseases (Mihov and Hasler 2016; Lindemann et al. 2015; Jaeschke et al. 2015; Pop et al. 2014; Levenga et al. 2011; Picconi and Calabresi 2014; Rascol et al. 2014; Zhang et al. 2014; Chung et al. 2015; Vaidya et al. 2013). Various human trials of mGluR5 allosteric modulators have been completed or are currently in progress to test their therapeutic relevance in L-DOPA-induced dyskinesia, Huntington's disease, Fragile-X syndrome and gastroesophageal reflux disorder (Youssef et al. 2018; Haass-Koffler et al. 2017; Tison et al. 2016; Reilmann et al. 2015; Berg et al. 2011; Zerbib et al. 2011).

The abundance of mGluR5 in various basal ganglia nuclei and its key regulatory functions at specific glutamatergic synapses throughout the basal ganglia circuitry has made it a prime therapeutic target for basal ganglia-related disorders such as Parkinson's disease and L-DOPA-induced dyskinesia (Masilamoni and Smith 2018; Rascol et al. 2014; Picconi and Calabresi 2014). Because of their enrichment in mGluR5 and their close link with the basal ganglia and motor cortices, the ventral motor and caudal intralaminar thalamic nuclei are additional targets through which mGluR5-related drugs could mediate their therapeutic antiparkinsonian and anti-dyskinetic effects in primates. However, despite evidence for cellular and neuropil expression of mGluR5 in these nuclei, very little is known about the subsynaptic localization and function of mGluR5 in the primate motor thalamus. Similarly, although imaging, immunohistochemical and in situ hybridization approaches have revealed variable levels of mGluR1 expression in the rodent and primate thalamus, details about its ultrastructural localization and function remain scarce.

To address this knowledge gap, the goal of the present study was to provide a detailed map of the cellular, subcellular and subsynaptic localization of mGluR5 in the ventral motor thalamus and the CM of rhesus monkeys. To determine if mGluR5 is preferentially associated with specific glutamatergic afferents, double immuno-electron microscopic studies using vGluT1 or vGluT2 as markers of cortical or sub-cortical glutamatergic terminals, respectively, was performed.

Materials and methods

Animals, perfusion and preparation of tissue

A total of three adult (1 male, 2 females) rhesus monkeys (*Macaca mulatta*) from the Yerkes Primate Center colony were used for this study. Animals were deeply anesthetized with an overdose of pentobarbital (100 mg/kg, i.v.) and perfused transcardially with cold oxygenated Ringer's solution followed by a fixative containing 4% paraformaldehyde and 0.1% glutaraldehyde in phosphate buffer (PB) (0.1 m; pH 7.4). All animal procedures were approved by the Institutional Animal Care and Use Committee at Emory University and conform to the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals (Garber et al. 2010). After perfusion, the brains were removed from the skull, cut in 10 mm-thick blocks in the coronal plane, and stored in cold phosphate-buffered saline (PBS; 0.01 M, pH 7.4) until sectioning. A vibrating microtome was used to cut the blocks into serial 60 µm-thick coronal sections that were collected in an antifreeze solution (1.4% NaH_2PO_4-H_20, 2.6% Na_2HPO_4-$7H_2O$, 30% ethylene glycol, 30% glycerol dissolved in distilled water) and stored in a − 20 °C freezer until further processing.

Primary antibodies

To localize mGluR1a and mGluR5, we used a commercially available affinity-purified rabbit polyclonal antiserum raised against the synthetic C-terminal peptides representing different amino acid sequences of rat mGluR1a conjugated to keyhole limpet hemocyanin with glutaraldehyde (Millipore, cat no. AB1551) and mGluR5 lysine added to the N-terminus (Millipore AB5675). To label glutamatergic terminals, we used commercially available affinity-purified guinea pig polyclonal and mouse monoclonal antisera raised against the synthetic COOH terminal peptide representing different amino acid sequences of rat vGluT1 (Millipore AB5905) and human vGluT2 (MAB tech (VGT2-6) Table 1), respectively. The specificity of these antibodies was tested in several laboratories including ours, by immunoblot analysis on proteins isolated from transfected cell lines and specific rat, rabbit and monkey brain regions (Marino et al. 2001; Raju et al. 2006; Kuwajima et al. 2004; Ge et al. 2014).

mGluR1a and mGluR5 labeling for light microscopy

Thalamic tissue sections containing the cerebellar- and basal ganglia-receiving ventral motor thalamic nuclei or the caudal intralaminar thalamic nuclei, as delineated in the

Table 1 Primary antibodies used in this study

Antibody	Immunogen	Manufacturer data	Dilution
Calbindin-D-28K	Bovine kidney calbindin-D	Sigma (C-9848)Mouse monoclonal	1: 4000
mGluR1a	Carboxy terminal peptide of rat mGluR1alpha conjugated to KLH with glutaraldehyde (PNVTYASVILRDYKQSSSTL)	Millipore (AB1551) Rabbit Polyclonal	1: 250
mGluR5	KLH-conjugated linear peptide corresponding to the cytoplasmic domain of mouse Metabotropic Glutamate Receptor 5	Millipore (AB5675) Rabbit Polyclonal	1: 5000
vGluT1	Synthetic peptide from rat VGLUT1 protein with no overlap to VGLUT2	Millipore (AB5905) Guinea Pig Polyclonal	1: 5000
vGluT2	Synthetic peptide (amino acids 560-578) coupled to KLH by the addition of an N-terminal cysteine	MAB tech (VGT2-6) Rabbit Polyclonal	1: 1000

rhesus monkey stereotaxic brain atlas (Paxinos et al. 1999) and in calbindin-immunostained adjacent sections (Calzavara et al. 2005), were removed from the anti-freeze solution and placed in phosphate-buffered saline (PBS, 0.01 M, pH 7.4). Then, they were immersed in sodium borohydride (1% in PBS) for 20 min followed by a pre-incubation for 1 h in a solution containing 1% normal goat serum (NGS), 0.3% Triton-X-100, and 1% bovine serum albumin (BSA) in PBS. Sections were then incubated overnight at room temperature (RT) in a solution containing rabbit anti-mGluR1a (1:250) or rabbit anti-mGluR5 (1:5000) in 1% NGS, 0.3% Triton-X-100 and 1% BSA in PBS. On the following day, sections were thoroughly rinsed in PBS and incubated in a PBS solution containing (secondary) biotinylated goat anti-rabbit IgGs, (1:200; Vector, Burlingame, CA) combined with 1% NGS, 0.3% Triton-X-100, and 1% BSA for 90 min at RT, then rinsed three times in PBS. Sections were exposed to an avidin–biotin–peroxidase complex (ABC; 1:100 Vector) for 90 min followed by rinses in PBS and TRIS buffer (0.05 M; pH 7.6). Sections were then incubated in a solution containing 0.025% 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO), 10 mM imidazole, and 0.005% hydrogen peroxide in TRIS buffer for 10 min at RT, rinsed with PBS, placed onto gelatin-coated slides, and coverslipped with Cytoseal XYL. Finally, the sections were analyzed using a Leica DMLB light microscope (Vienna, Austria) and photographed using a ScanScope light microscope (Aperio Technologies, Vista, CA).

Single immunoperoxidase labeling for EM

Three tissue sections per animal containing the ventral motor thalamus or the caudal intralaminar nuclei were processed for the EM immunoperoxidase localization of mGluR1a and mGluR5. Sections were treated with a 1% sodium borohydride solution, placed in a cryoprotectant solution (PB 0.05 M, pH 7.4, 25% sucrose, and 10% glycerol), frozen at − 80 °C for 20 min each before being returned to PBS-based solutions with decreasing gradients of cryoprotectant, and last, washed thoroughly with PBS. Sections processed for immunoperoxidase were incubated the same way as for light microscopy except that no Triton-X-100 was used in any solutions and that the incubation lasted 48 h at 4 °C. After DAB exposure, the tissue was rinsed in PB (0.1 M, pH 7.4) and treated for 20 min with 1% OsO4, returned to PB, and then dehydrated with increasing concentrations of ethanol. To increase the tissue contrast at the electron microscope level, 1% uranyl acetate was added to the 70% ethanol solution for 35 min in the dark. After alcohol dehydration, sections were placed in propylene oxide and embedded in epoxy resin (Durcupan, Fluka, Buchs, Switzerland) for at least 12 h, mounted onto slides, and placed in a 60 °C oven for 48 h (Smith and Bolam 1991). Tissue samples from the pallidal- or cerebellar-receiving regions of the ventral motor nuclei and from the centromedian (CM) nucleus were cut out of large, resin-embedded sections and fixed onto resin blocks, before being cut into 60 nm ultrathin sections (Leica Ultracut T2). These sections were mounted onto Pioloform-coated copper grids, stained with a lead citrate for 5 min and then examined with an electron microscope (EM; model 1011, Jeol, Peabody, MA). Digital micrographs of immunoreactive elements were collected with a Gatan CCD camera (Model 785; Warrendale, PA) controlled by Digital Micrograph software (version 3.11.1).

Immunogold labeling for EM

The immunogold-stained sections were used to elucidate the specific localization of mGluR5 labeling in relation to synaptic and non-synaptic sites along the plasma membrane of thalamic neurons. Sections were treated with sodium borohydride and processed with the cryoprotectant protocol described above. This was followed by rinses in PBS and pre-incubation for 30 min in a PBS solution containing 5% dry milk. Sections were then rinsed in a TRIS-buffered saline (TBS)-gelatin buffer (0.02 M, 0.1% gelatin, pH 7.6) and incubated with the primary antibody solution prepared with 1% dry milk in TBS-gelatin buffer for overnight at RT. One day later, sections were rinsed in TBS-gelatin buffer and then treated for 2 h at room temperature with the secondary antibody solution (goat anti-rabbit Fab′ fragments conjugated with 1.4 nm gold particles 1:100; Nanoprobes,

Yaphank, NY) prepared with 1% milk in TBS-gelatin buffer. Sections were then washed in TBS-gelatin buffer and 2% sodium acetate buffer before incubation with the HQ Silver Kit (Nanoprobes) for 4–10 min to increase gold particle sizes to 30–50 nm through silver intensification. The sections were then treated according to the same protocol of osmification, dehydration, embedding, and tissue selection as used for the tissue processed according to the pre-embedding immunoperoxidase procedure, including the following changes: (1) the tissue was kept in 0.5% OsO_4 for 10 min instead of 20 and (2) the tissue was stained with 1% uranyl acetate for 10 min instead of 35 min.

Double labeling for vGluT1, vGluT2 and mGluR5

To identify the glutamatergic terminals associated with mGluR5 receptor labeling, we used a double immunocytochemical approach to localize mGluR5 labeling with vGluT1 or vGluT2 immunostaining, identified as largely specific markers of cortical or subcortical glutamatergic terminals, respectively, in the motor and intralaminar thalamic regions under study (Raju et al. 2006). It is also worth noting that a small subset of thalamic cells co-express vGluT1 and vGluT2 mRNA in rats (Barroso-Chinea et al. 2007). However, because there is no evidence for VGluT1 and VGluT2 protein co-localization in thalamostriatal terminals (Lacey et al. 2005; Fujiyama et al. 2006; Raju et al. 2008), the significance of this mRNA co-localization remains unclear. In these double labeled sections, vGluT1 or vGluT2 immunolabeling was localized with DAB, whereas mGluR5 immunoreactivity was detected with pre-embedding immunogold. The tissue was treated with the same methods as for the single electron microscopic immunogold labeling, but the antibodies for vGluT1 and mGluR5 or vGluT2 and mGluR5 were pooled together for overnight incubation at RT. The concentrations of the secondary antibodies, ABC, and DAB as well as the embedding procedures were the same as those used for the single immunogold labeling. Afterward, sections were washed in TBS-gelatin and incubated for 1.5 h in a 1:100 ABC solution. This was followed by washes in TBS-gelatin and TRIS buffer before a 10 min incubation in DAB (see above) to localize vGluT1 or vGluT2. After many washes in TBS-gelatin, sections were processed for electron microscopy as described above for immunogold single labeling.

As controls, the vGluT1 and mGluR5 or vGluT2 and mGluR5 antibodies were omitted in turn from the incubation solution, whereas the rest of the procedure remained the same. This resulted in a lack of labeling corresponding to the omitted antibodies (i.e. the tissue was devoid of DAB staining in the absence of vGluT1 or vGluT2 antibodies, whereas omission of the mGluR5 antibodies resulted in a complete lack of gold-particle labeling). Approximately 50 micrographs of randomly selected tissue areas that contained both immunoperoxidase and immunogold labeling in the same field of view were taken from each animal for each receptor combination at 60,000×. From each of these sections, we categorized the ultrastructural features of the different immunoreactive elements labeled with gold, peroxidase or both. Micrographs of gold-labeled dendrites were analyzed for total length of dendritic plasma membrane and total length of synaptic and perisynaptic plasma membrane by using the image J software (NIH).

Analysis of electron microscopy material

To compare the overall distribution of mGluR1 and mGluR5 labeling in the ventral motor thalamic regions and CM, 50 digital micrographs of randomly encountered mGluR1a- and mGluR5-labeled neuronal elements were captured in each animal at 40,000× (Orius 78; Gatan, Inc., Pleasanton, CA, USA), yielding 764 μm^2 of tissue analyzed per animal. Elements labeled with the peroxidase deposit were categorized as dendrites (projection neurons or interneurons), axon terminals and glia on the basis of ultrastructural features described by Peters et al. (1991). Dendrites of thalamic interneurons were characterized as vesicle-filled neuronal processes post-synaptic to axon terminals (Montero and Scott 1981; Montero and Singer 1984; Hamos et al. 1985). The density of labeled elements was calculated by dividing the number of elements labeled by the total area of tissue examined.

In mGluR5-immunostained sections with the pre-embedding immunogold method, gold particles were categorized as either intracellular or plasma membrane (PM)-bound depending on their localization relative to the PM. To be categorized as PM-bound, gold particles had to be in contact with the membrane; all other particles were considered intracellular. PM-bound gold particles were further classified into two categories: perisynaptic (touching or within a 20-nm range of the edges of postsynaptic specializations) or extrasynaptic (on the PM, but not associated with synapses) (Blackstad et al. 1990). The percentages of total gold particles in each of those categories were then calculated for each animal, and the mean number of perisynaptic or extrasynaptic gold particles was calculated across the number of animals and presented as a bar histogram. Data were analyzed for significant differences, in SigmaStat software, by two-way repeated-measures ANOVAs and Tukey's post hoc test. The percentage of PM-bound gold particles was compared across each neuronal element.

For double labeling, approximately 50 micrographs of randomly selected tissue areas that contained both immunoperoxidase and immunogold labeling in the same field of view were taken from each animal for each receptor combination at 60,000×. From each of these sections, we categorized the

ultrastructural features of the different immunoreactive elements labeled with gold, peroxidase or both.

Results

Light-microscopic observations

At the light microscopic level, we used differential intensity of calbindin immunostaining to help delineate borders between the calbindin-enriched basal ganglia-receiving nuclei (VApc/VAmc) and the calbindin-poor cerebellar-receiving region (VLp) (Fig. 1a, b). The lack of calbindin labeling was also used to delineate the borders of the CM/Pf caudal intralaminar complex (Fig. 1c). Although both mGluR1a and mGluR5 immunoreactivity was expressed throughout the full extent of the ventral motor and caudal intralaminar nuclei, the overall intensity of mGluR5 immunostaining was stronger than that of mGluR1a (compare Fig. 1a'–c' with Fig. 1a''–c''). At the cellular level, both mGluR1a and mGluR5 labeling was largely associated with neuronal cell bodies and neuropil elements which, at

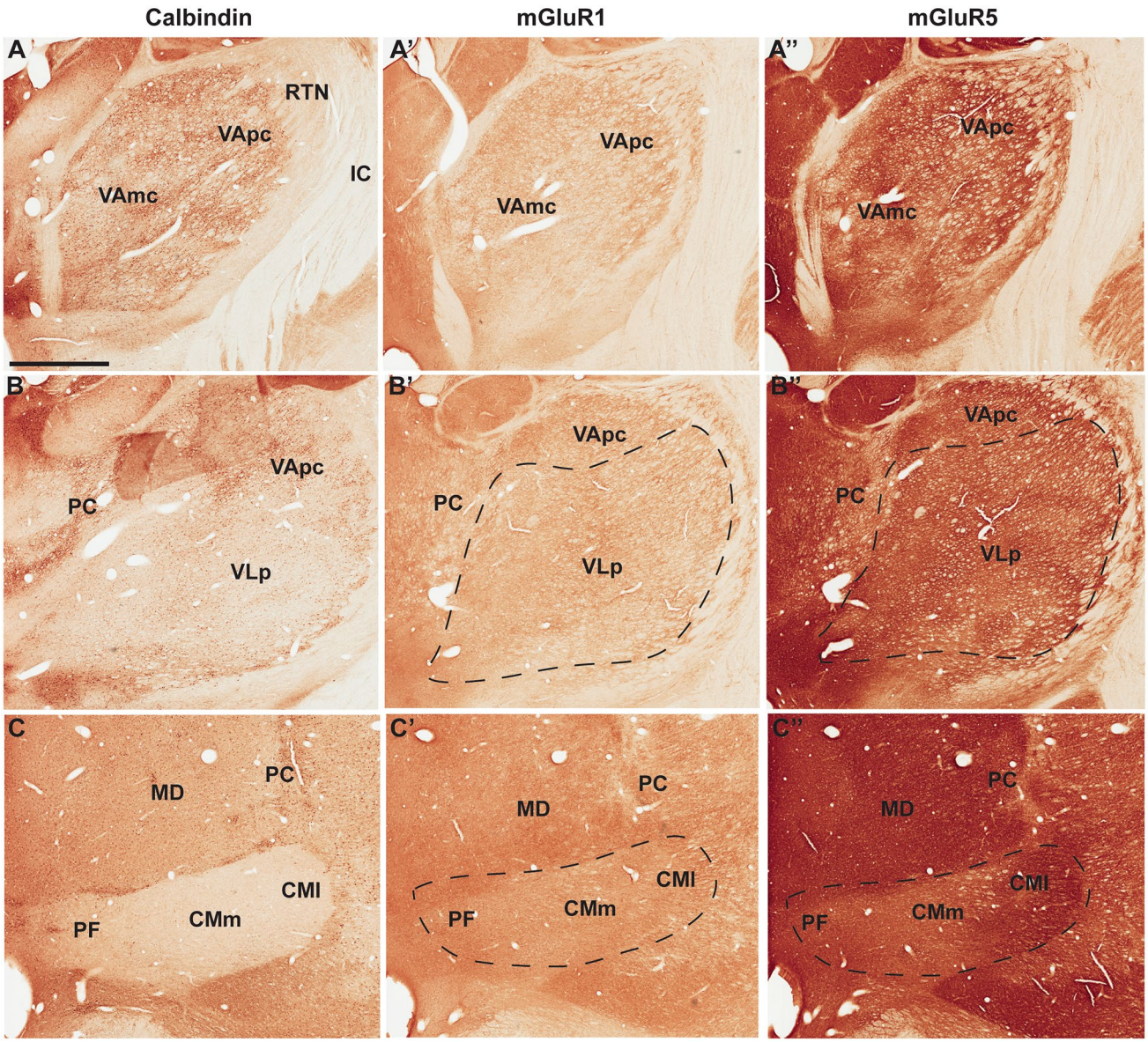

Fig. 1 Photomicrographs of adjacent calbindin-, mGluR1a- and mGluR5-immunostained coronal sections at the level of VA-VL and CM regions of the monkey thalamus. Calbindin immunostaining was used to delineate the borders of VApc, VLp and CM nuclei. Scale bar in **a**: 2 mm (applies to all panels). *VApc* ventral anterior nucleus parvocellularis, *VAmc* ventral anterior nucleus magnocellularis, *CMm* centromedian thalamic nucleus medial, *CMl* centromedian thalamic nucleus lateral

high magnification, included numerous small punctate and beaded structures (Fig. 1). The overall distribution of labeling with the two antisera was homogeneous between the VApc/Vamc and the VLp (Fig. 1a'b', a", b"). At the level of the CM/Pf, the lateral third of the CM (CMl) displayed a stronger level of mGuR5 immunoreactivity than the medial CM (CMm) and the Pf (Fig. 1c', c").

EM immunoperoxidase localization of group 1 mGluRs in the VApc and CM

At the EM level, the immunoperoxidase labeling for either mGluR1a or mGluR5 in VApc and CM was most commonly found in dendritic profiles contacted by putative unlabeled glutamatergic terminals (Fig. 2a, b, e). In some instances, the peroxidase deposit was closely associated with the postsynaptic density of the asymmetric synapses (Fig. 2a), but in most cases, it was diffusely distributed throughout the labeled structures (Figs. 2, 3). A small number of glial processes and axon terminals were labeled for either receptor subtypes (Figs. 2e, 3e). The immunoreactive glial processes displayed an astrocytic morphology, i.e. they were usually thin, had an irregular shape, followed a tortuous course to fill space between neuronal elements, and were not aggregated in bundles (Figs. 2d, 3b). The labeled dendrites were categorized as originating from projection neurons (PN) or interneurons (IN) based on their intracellular content. IN dendrites contained synaptic vesicles, often received asymmetric synaptic inputs and occasionally formed dendro-dendritic synapses with neighboring unlabeled dendrites (Ralston 1971; Hamos et al. 1985; Ohara et al. 1989; Jones 2007), while PN dendrites were devoid of synaptic vesicles and did not act as the pre-synaptic element of dendro-dendritic synaptic complexes (Smith et al. 1987). In both VApc and CM, the general distribution of mGluR1a or mGluR5 labeling was quite similar, i.e. the number of PN dendrites far outnumbered (4–6 times larger) other immunoreactive profiles including axon terminals, glia and IN dendrites (Figs. 2e, 3e).

EM immunogold localization of mGluR5 in the VApc, VLp and CM

To study the subcellular and subsynaptic localization of mGluR5 labeling, we used the pre-embedding immunogold method, which offers a higher level of spatial resolution than the immunoperoxidase method (Galvan et al. 2006). This part of the study was focused solely on mGluR5 because the immunogold signal for mGluR1a was below a reliable detection level. Overall, the pattern of mGluR5 immunogold labeling amongst neuronal elements was similar to that found in the immunoperoxidase-stained sections of VApc, VLp and CM (Fig. 3e), i.e. gold labeling was localized predominantly in PN dendrites of various sizes, with additional

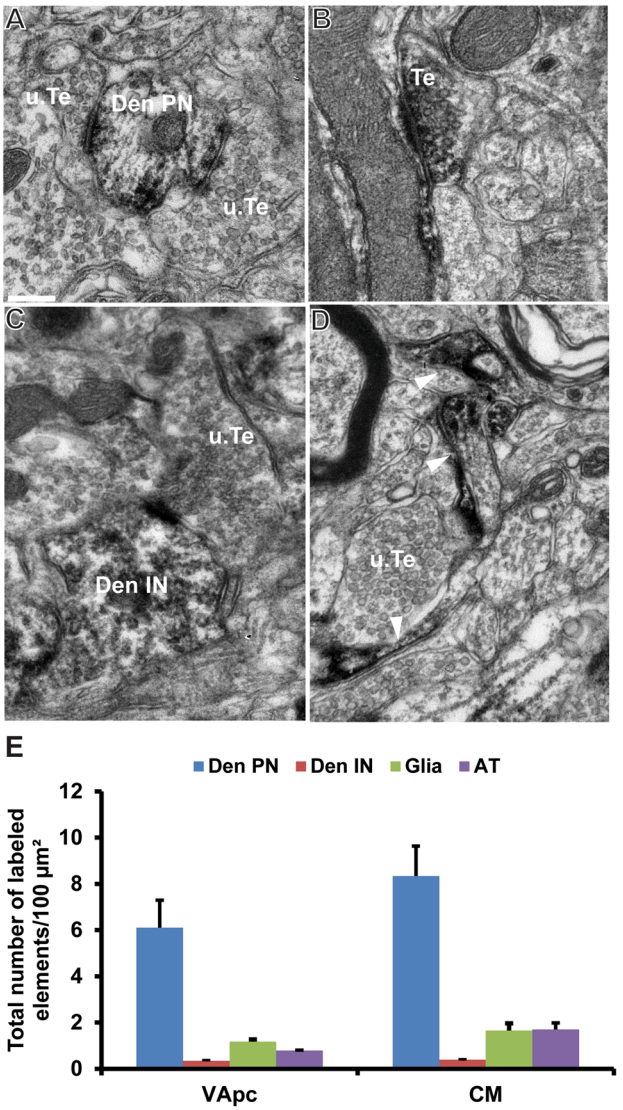

Fig. 2 Immunoperoxidase localization of mGluR1a in the monkey VApc and CM. **a**–**d** Electron micrographs of a mGluR1a-immunoreactive dendrite of a projection neuron (Den PN; **a**), an axon terminal (Te; **b**), a dendrite of an interneuron (Den IN; **c**) and a glial process (**d**). Unlabeled terminals (u.Te) are seen in the neuropil. Scale bar in **a**: 250 nm (applies to all panels). **e** Relative abundance of mGluR1a-immunoreactive elements in the neuropil of the monkey VApc and CM. Values are shown as mean ± SD density of labeled elements in each category

lighter labeling in IN dendrites, axons, terminals and glia. In labeled dendrites, 75.3% and 80.1% gold particles were localized on the PM of PN and IN dendrites, respectively, whereas about 20–25% gold particles were localized in the intracellular compartment (Fig. 4). Of the total PM-bound gold particles, more than 95% were extrasynaptic (Figs. 4b, d, f), while the remaining particles were perisynaptic to asymmetric postsynaptic specializations (Fig. 4c, f). Only 2 PM-bound gold particles were detected in the main body

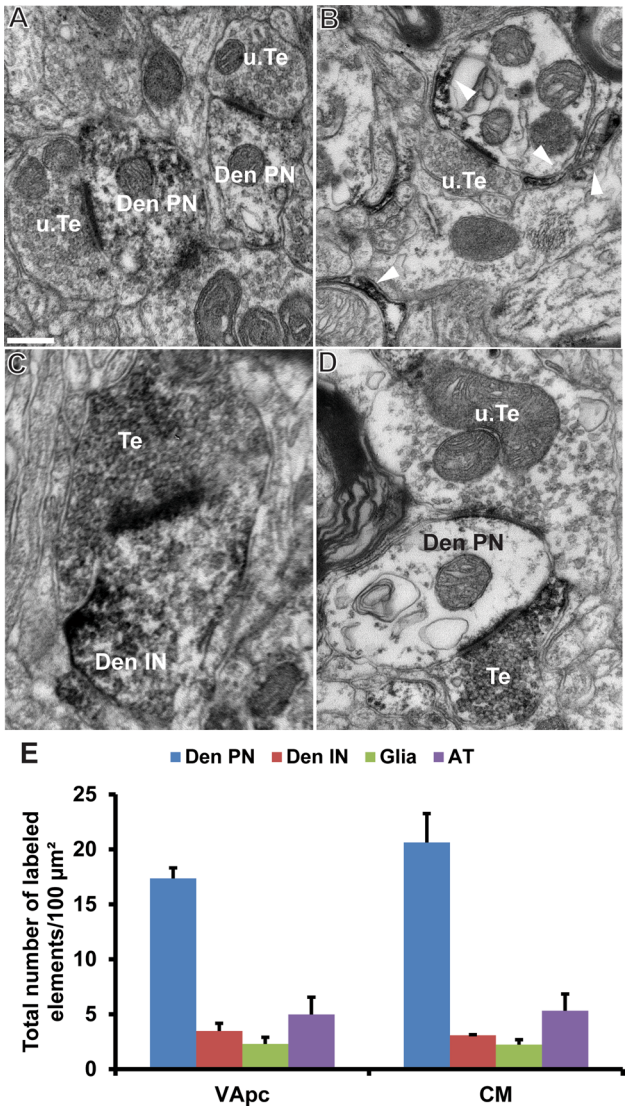

Fig. 3 Immunoperoxidase localization of mGluR5 in the monkey VApc and CM. **a**–**d** Electron micrographs of a mGluR5-immunoreactive dendrite of projection neuron (Den PN; **a**), a glial process (**b**), an interneuron dendrite (Den IN; **c**) and axon terminals (Te; **c**, **d**). Unlabeled terminals (u.Te) are also shown in the neuropil (**a**, **b**, **d**). Scale bar in **a**: 250 nm (applies to all panels). **e** Density of mGluR5-immunoreactive elements in the monkey VApc and CM. Values are mean ± SD density of labeled elements in each category

of synaptic junctions, suggesting very scarce "synaptic" mGluR5 labeling in VApc, VLp and CM.

Perisynaptic labeling of mGluR5 at cortical and sub-cortical glutamatergic synapses

To determine whether the perisynaptic mGluR5 labeling is preferentially associated with cortical or sub-cortical glutamatergic terminals, double pre-embedding immunolabeling for vGLuT1 (marker of cortical glutamatergic terminals) or vGluT2 (marker of sub-cortical glutamatergic terminals) with mGluR5 was carried out on thalamic tissue from the VApc, VLp and CM. Consistent with previous studies in rats (Lacey et al. 2005; Fujiyama et al. 2006; Raju et al. 2006; Moss and Bolam 2008) and monkeys (Raju et al. 2008), vGluT1 and vGluT2 immunoperoxidase labeling was concentrated within axons and axon terminals forming asymmetric synapses (Figs. 5, 6). From these double labeled sections, 50 micrographs of vGluT1- or vGluT2-labeled terminals in contact with mGluR5-immunoreactive dendrites were taken in each nucleus. The total number of gold particles in each postsynaptic dendrite was counted. The PM-bound gold particles were categorized as perisynaptic or extrasynaptic in relation to the asymmetric synapses formed by the vGluT-labeled terminals. To determine whether the PM-bound gold particles displayed a preferential association with the perisynaptic or extrasynaptic microdomains of the PM, we normalized the percentage of gold particles bound to the extrasynaptic or perisynaptic PM domains to the relative proportion of the dendritic PM that contributed to these microdomains (Figs. 5d–f, 6d–f). Following such analysis, the relative labeling density of perisynaptic mGluR5 immunogold labeling at synapses formed by VGluT1 and VGluT2-containing terminals was significantly higher than would be expected from a random distribution across all three nuclei (Figs. 5f, 6f).

Discussion

The results of this study provide the first description of the cellular, subcellular and subsynaptic localization of group I mGluRs in the ventral motor thalamus and CM of primates. Four major conclusions can be drawn from these data. First, both mGluR1a and mGluR5 immunostaining is homogeneously expressed in the basal ganglia- and cerebellar-receiving regions of the ventral motor thalamus in monkeys. Second, mGluR5, but not mGluR1a, immunoreactivity is more strongly expressed in the lateral than the medial parts of the CM, suggesting a preferential association with thalamocortical over thalamostriatal neurons in the primate CM (Smith and Parent 1986; Sadikot et al. 1992). Third, mGluR1a and mGluR5 are expressed postsynaptically in dendrites of projection neurons and GABAergic interneurons in the basal ganglia- and cerebellar-receiving regions of the ventral motor thalamus and in CM. Fourth, the plasma membrane-bound mGluR5 immunoreactivity is preferentially expressed perisynaptically at the edges of asymmetric synapses formed by cortical and sub-cortical glutamatergic afferents. Overall, group I mGluRs, in particular mGluR5 are located to subserve a modulatory role of cortical and subcortical glutamatergic inputs to projection neurons and interneurons in the ventral motor thalamus and CM of rhesus monkeys.

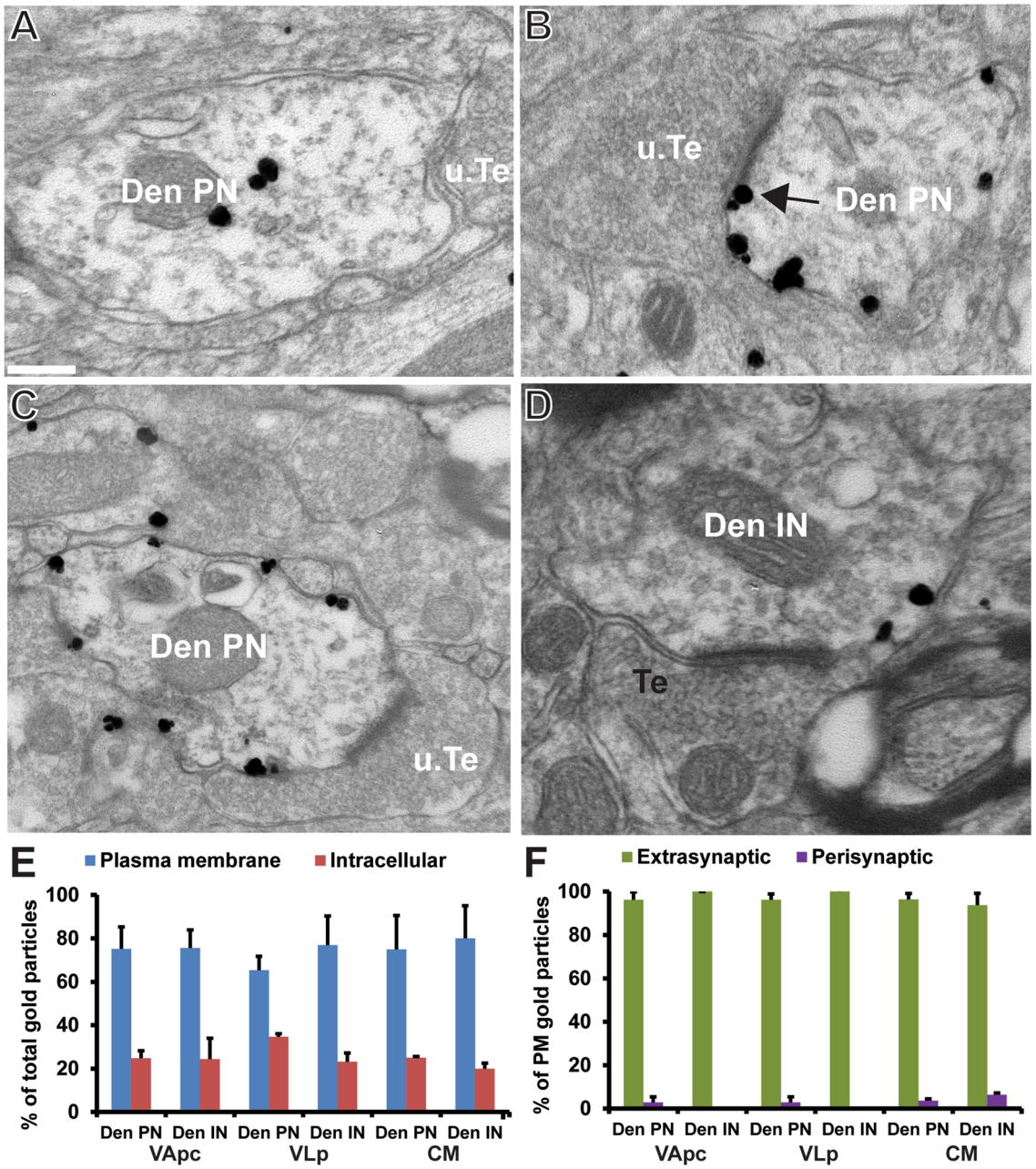

Fig. 4 Electron micrographs showing the subcellular distribution of mGluR5 **a**–**d** immunogold labeling in the monkey VApc, VLp and CM. Representative photomicrographs of **a** intracellular, **b** perisynaptic (black arrow), **c** plasma membrane-bound extrasynaptic labeling in a dendrite of projection neuron (PN), and **d** extrasynaptic labeling on a dendrite of an interneuron (IN). Scale bar in **a**: 250 nm (applies to all panels). **e** Percentage of plasma membrane-bound (PMB) versus intracellular gold particles labeling in VApc, VLp and CM. Values are mean ± SD. **f** Relative percentages of extrasynaptic versus perisynaptic PMB gold particle labeling in dendrites of projection neurons and interneurons in the VApc, VLp and CM

Regional group I mGluRs localization in the primate thalamus

Our light microscopic data of group I mGluRs immunoreactivity in the monkey thalamus are consistent with previous immunohistochemical and in situ hybridization data in rodents and monkeys (Testa et al. 1994; Abe et al. 1992; Shigemoto et al. 1992; Ouattara et al. 2010), and with PET imaging results in humans (Toyohara et al. 2013; Varnas et al. 2018; Yamasaki et al. 2014). These various studies concur that both receptor subtypes are expressed in the mammalian thalamus, albeit to a different degree. In situ hybridization studies have reported a stronger expression of mGluR1 than mGluR5 mRNA throughout the

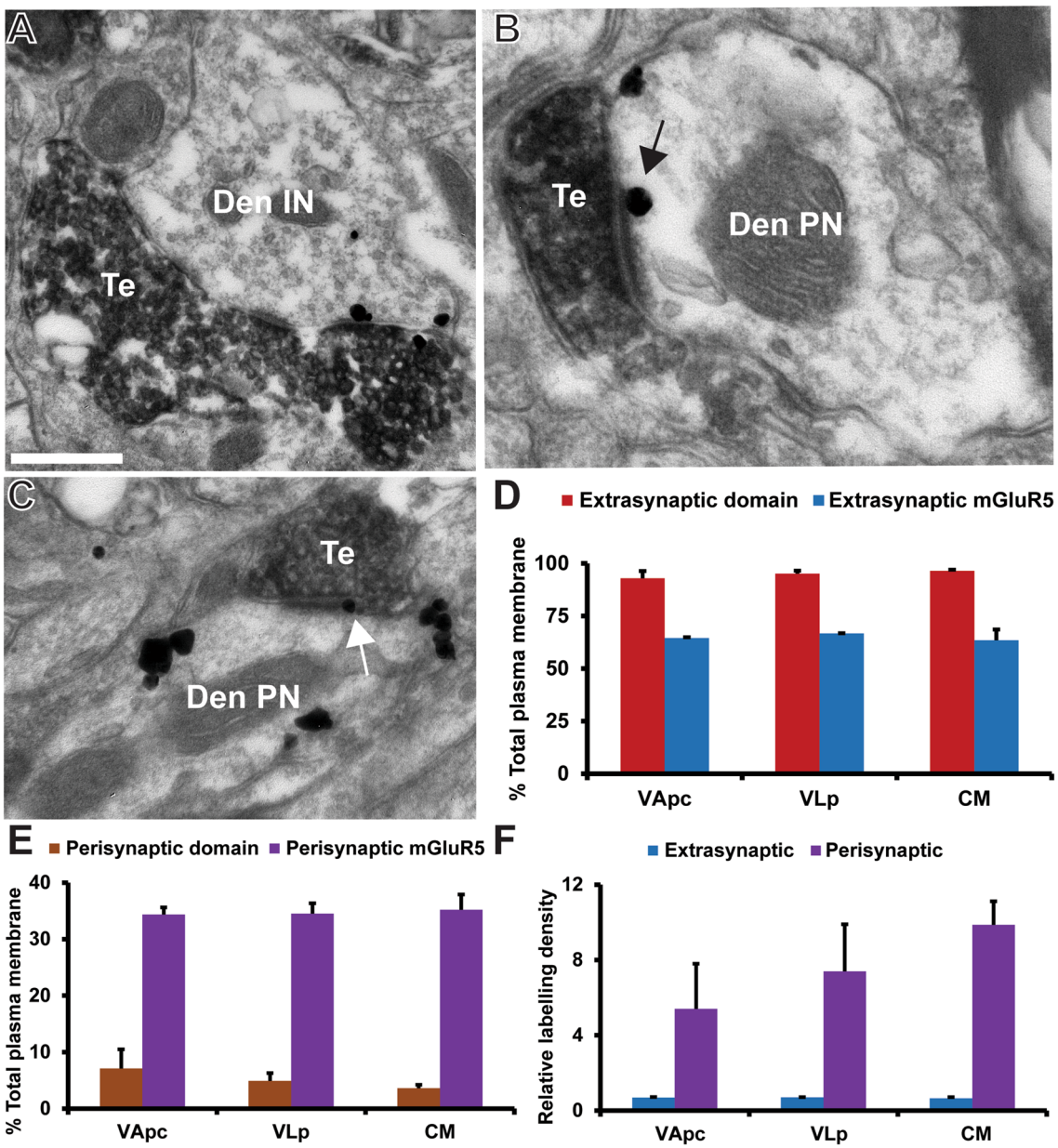

Fig. 5 **a**–**c** Electron micrographs of thalamic tissue double immunostained for mGluR5 (immunogold) and VGLuT1 (immunoperoxidase) at the level of VApc, VLp and CM showing vGluT1-positive terminals forming asymmetric synapses with mGluR5-labeled dendrites. Note synaptic and perisynaptic mGluR5 labeling (arrows). Scale bar in **a**: 350 nm (applies to all panels). **d**, **e** Comparison between the percentages of mGluR5 labeling at extrasynaptic (**d**) and perisynaptic (**e**) sites and the proportion of total plasma membrane (PM) occupied by these two sub-synaptic domains on Den of thalamic cells. **f** Relative density of extrasynaptic and perisynaptic mGluR5 immunogold labeling on the Den of thalamic neurons when values are normalized to the amount of PM devoted to these specific sub-synaptic domains. The normalized values were calculated as the percentage of labeling for mGluR5 in a specific sub-synaptic domain divided by the dendritic membrane that contributes to that domain along the PM. The relative density of perisynaptic mGluR5 labeling is larger than the density of extrasynaptic labeling when values are normalized to the amount of PM devoted to each sub-synaptic domain

rodent thalamus (Testa et al. 1994; Shigemoto et al. 1992). Although similar mRNA localization studies have not been achieved in the primate thalamus, imaging studies indicate that mGluR5 ligands are strongly expressed in the primate thalamus (Varnas et al. 2018; Andersson et al. 2013). Our findings demonstrate a stronger expression of mGluR5 than mGluR1a immunoreactivity in the ventral motor thalamus and CM of rhesus monkeys. Whether this differential immunostaining intensity is reflective of a genuine larger mGluR5 than mGluR1a protein expression or a higher sensitivity of mGluR5 than mGluR1a antibodies for their respective antigens in thalamic cells remain unclear. Asides from their

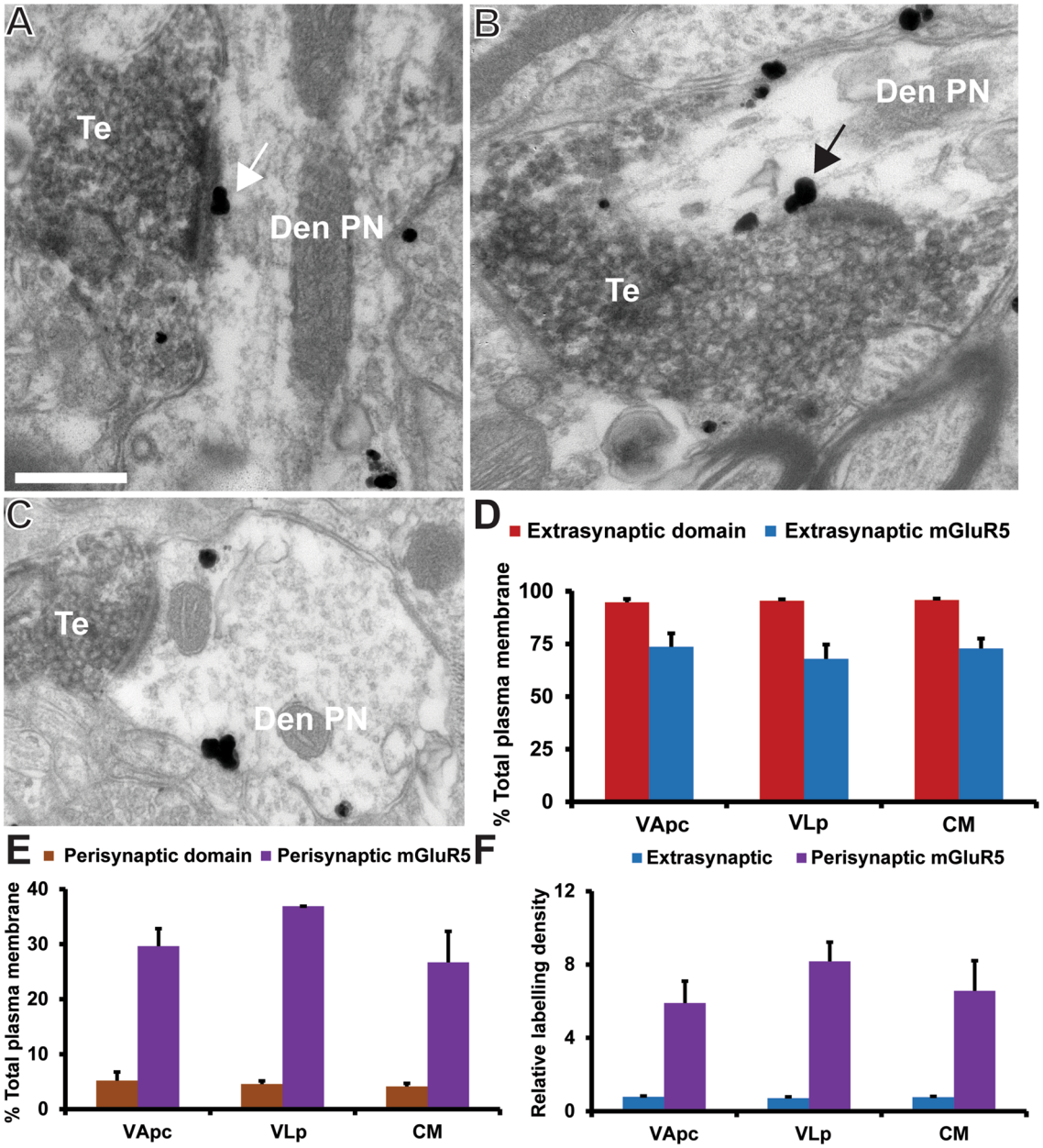

Fig. 6 **a**–**c** Electron micrographs of thalamic tissue double immunostained for mGluR5 (immunogold) and VGLuT2 (immunoperoxidase) at the level of VApc, VLp and CM showing vGluT2-positive terminals forming asymmetric synapses with mGluR5-labeled dendrites. Note synaptic and perisynaptic mGluR5 labeling (arrows). Scale bar in **a**: 350 nm (applies to all panels). **d**, **e** Comparison between the percentages of mGluR5 labeling at extrasynaptic (**d**) and perisynaptic (**e**) sites and the proportion of total plasma membrane (PM) occupied by these two sub-synaptic domains on Den of thalamic cells. **f** Relative density of extrasynaptic and perisynaptic mGluR5 immunogold labeling on the Den of thalamic neurons when values are normalized to the amount of PM devoted to these specific sub-synaptic domains. The normalized values were calculated as the percentage of labeling for mGluR5 in a specific sub-synaptic domain divided by the dendritic membrane that contributes to that domain along the PM. The relative density of perisynaptic mGluR5 labeling is larger than the density of extrasynaptic labeling when values are normalized to the amount of PM devoted to each sub-synaptic domain

relative abundance, our findings provide a strong foundation for the possible co-existence of both group I mGluR subtypes in the monkey motor thalamus and CM, suggesting that mGluR1 and mGluR5 might functionally interact or mediate different functions in thalamic cells. Although the differential role of mGluR1 or mGluR5 in regulating neuronal activity in the ventral motor thalamus and CM is unknown, findings from other brain regions, like the globus pallidus, where both receptors co-exist at the single cell level, have revealed specific regulatory cross-talks between

the two receptor subtypes which allow mGluR5 to regulate the desensitization of mGluR1 (Poisik et al. 2003). In the hippocampus, co-localized mGluR1 and mGluR5 play distinct roles in regulating excitability of CA1 neurons (Mannaioni et al. 2001). Our data set the anatomical foundation to explore such interactions in thalamocortical and GABAergic interneurons in the primate ventral motor thalamus and CM.

Although the overall expression of either group I mGluR subtypes was quite homogeneous throughout the basal ganglia- and cerebellar-receiving regions of the motor thalamus, the expression of mGluR5 immunostaining was higher in the lateral than the medial parts of CM. The significance of this stronger mGluR5 expression in lateral CM remains to be established. It is noteworthy that the lateral moiety of CM harbors predominantly thalamocortical neurons, while the medial CM is largely made up of thalamostriatal neurons that project to the putamen (Smith and Parent 1986; Sadikot et al. 1992). Thus, our findings set the stage for a possible differential expression and regulatory function of mGluR5 in thalamocortical vs thalamostriatal neurons in the primate CM.

Subcellular localization of group I mGluRs in the primate motor thalamus and CM

Overall, the subcellular pattern of mGluR1a or mGluR5 expression was quite similar in VApc and CM of rhesus monkeys, i.e. the bulk of immunoreactive elements for either receptor subtypes was accounted for by dendrites of thalamocortical neurons with moderate to low prevalence of interneuron dendrites, glial cell processes and axon terminals. As expected, based on the stronger intensity of mGluR5 than mGluR1a labeling through the thalamus, the density of mGluR5-positive elements was substantially larger than that of mGluR1a-immunorecative neuronal and glial profiles in both VApc and CM. Using the pre-embedding immunogold method, we could further assess the spatial localization of mGluR5 within immunoreactive dendrites, and found that most intra-dendritic mGluR5 immunoreactivity was closely apposed to the plasma membrane of labeled dendrites. Of these plasma membrane-bound receptors, a significant proportion was categorized as peri-synaptic because they were found at the edges of the asymmetric post-synaptic densities of glutamatergic synapses. This pattern is consistent with previous reports on the localization of group I mGluRs in various brain regions, including sensory thalamic nuclei in rodents (Baude et al. 1993; Lujan et al. 1996; Nusser et al. 1994; Ottersen and Landsend 1997; Liu et al. 1998). Consistent with reports from other brain regions, the perisynaptic localization of mGluR5 in the primate thalamus suggests that the mGluR5 activation relies on spillover of neurotransmitter from the synaptic cleft of glutamatergic synapses (Pal 2018; Kullmann et al. 1999; Govindaiah et al. 2012b; Drew et al. 2008; Zhang and Sulzer 2003; Galvan et al. 2006). Based on our double immuno-electron microscopy data, we conclude that both cortical (vGluT1-positive) and sub-cortical (vGluT2-positive) glutamatergic terminals mediate some of their physiological effects through mGluR5. Although the exact source(s) of vGluT2 terminals were not determined in this study, the deep cerebellar nuclei likely account for most of them in VLp, the main target of ascending cerebellar projections in monkeys (Strick 1985; Matelli et al. 1989; Sakai et al. 1996; Mason et al. 2000; Sakai et al. 2000; Dum et al. 2002; Evrard and Craig 2008). However, other potential sources, such as the pedunculopontine nucleus, reticular formation, etc. cannot be ruled out, particularly in VApc and CM (Kobayashi and Nakamura 2003). Thus, it appears that mGluR5 is located to subserve a modulatory role on the corticothalamic and cerebellothalamic glutamatergic transmission in the primate ventral motor thalamus and CM. Although much remains to be known about the role of mGluR5 in the motor thalamus and CM, in vitro slice studies in rodents have suggested modulatory functions of mGluR5 on neuronal activity in the reticular thalamic nucleus and lateral geniculate nucleus (LGN) (Sherman 2014). Activation of group I mGluRs by either synaptic inputs or exogenous agonists trigger changes in membrane potential (Cox and Sherman 1999), oscillatory activity (Long et al. 2004; Blethyn et al. 2006), or long-term plasticity at electrical synapses interconnecting thalamic reticular neurons (TRN) (Landisman and Connors 2005). It has also been shown that activation of mGluR1 and mGluR5 on GABAergic interneurons in the LGN increase GABA release onto synaptic and extrasynaptic GABA-A receptors on thalamocortical neurons (Errington et al. 2011; Govindaiah et al. 2012a, b). In the dorsal lateral geniculate nucleus (dLGN), the interneurons can alter the temporal precision of retinogeniculate inputs (Blitz and Regehr 2005; Crunelli et al. 1988), regulate the receptive field properties of TC neurons, (Sillito and Kemp 1983; Berardi and Morrone 1984; Holdefer et al. 1989), and dynamically sculpt thalamic network activity (Lorincz et al. 2009). Thus, through modulation of these interneurons, mGluR5 can play a critical role in regulating visual processing. Our EM findings showed that mGluR1a and mGluR5 are also expressed on vesicle-filled dendrites of GABAergic interneurons in ventral motor and CM thalamic nuclei, thereby suggesting that the regulatory effects of mGluR5 on the motor thalamocortical and thalamostriatal systems can be mediated directly or indirectly via GABAergic interneurons.

Glial expression of mGluR1a and mGluR5 in the ventral motor thalamus and CM

Our findings indicate that the two group I mGluRs are expressed in glial cells in the monkey motor thalamus and

CM. Glial expression of group I mGluRs, in particular mGluR5, has been reported in various brain regions, including basal ganglia and other thalamic nuclei (Pirttimaki et al. 2011; Parri et al. 2010; Kuwajima et al. 2004; Paquet and Smith 2003). In some regions, this glial expression is developmentally regulated, being significantly much more profuse in young than adult age (Hubert and Smith 2004). One of the main function suggested for glial mGluR5 is to regulate glia-neuronal communication through Ca^{++}-dependent gliotransmitter release at tripartite glutamatergic synases (Pirttimaki and Parri 2012; Perea and Araque 2005). Stimulation of intracellular Ca^{++} waves in glial cells results in neuronal calcium spikes [for reviews, see Parpura et al. (1994); Araque et al. (1999); Verkhratsky et al. (1998)] which, when produced by electrical or mechanical stimulation, are sensitive to group I mGluR drugs (Araque et al. 1998). Although this glutamate-dependent neuroglial calcium signaling has generated significant interest, it is likely to be more critical in young that adult brains (Sun et al. 2013). Observations made in slices of the somatosensory ventrobasal (VB) thalamus of young rats confirm and extend these findings, showing that astrocytic group I mGluRs activation plays a dual role in this thalamic region, depending on the strength and pattern of afferent inputs (Pirttimaki and Parri 2012; Parri et al. 2010). Thus, although the developmental expression and role of glial group I mGluRs expression in the ventral motor nuclei and CM has not been examined, it is tempting to speculate that its function in glia-neuronal communication may be more significant in young than adult thalami, but this remains to be directly tested.

Perisynaptic mGluR5 labeling at cortical and sub-cortical glutamatergic synapses

The present immunogold data revealed three major structural features related to the subsynaptic localization of mGluR5 in the monkey motor thalamus and CM: (1) Over 80% gold particle labeling was bound to the plasma membrane of small-, medium- and large-sized dendrites of thalamocortical neurons in VApc, VLp and CM. This preferential plasma membrane bound localization of mGluR5 is consistent with rodent data from other thalamic nuclei (Liu et al. 1998), but different from the mGluR5 labeling pattern described in various basal ganglia nuclei, in which a significant proportion of gold labeling was found in intracellular compartments of labeled neuronal structures (Hanson and Smith 1999; Smith et al. 2000; Marino et al. 2001; Kuwajima et al. 2004; Hubert et al. 2001; Smith et al. 2001). Whether this is indicative of a differential rate of plasma membrane trafficking and internalization of mGluR5 between thalamic and BG nuclei, or evidence for a selective intracellular function of mGluR5 in BG nuclei, remains unclear, but deserves further consideration. (2) A large pool of plasma membrane-bound mGluR5 was located extrasynaptically on dendrites of thalamocortical neurons in VApc, VLp and CM. This pattern is reminiscent of the mGluR5 distribution found in other brain regions including most BG nuclei (Hanson and Smith 1999; Smith et al. 2000; Marino et al. 2001; Kuwajima et al. 2004; Hubert et al. 2001; Smith et al. 2001). In fact, most G-protein-coupled receptors are located extrasynaptically throughout the CNS (Smith et al. 2000, 2001; Yung et al. 1995; Beczkowska et al. 1997; Rodriguez et al. 1999). Such a non-synaptic localization raises some interesting questions about the sources and mechanisms of activation of these receptors. As discussed in in detail previous studies, transmitter spillover from glutamatergic synapses and/or glial release of glutamate should be considered as potential sources of activation of these non-synaptic receptors (Pal 2018; Galvan et al. 2006; Smith et al. 2000, 2001). The fact that tetanic stimulation of glutamatergic afferents and/or blockade of glutamate transporters is necessary to generate a group I mGluR-mediated postsynaptic EPSC is in line with this possibility (Conn and Pin 1997; Niswender and Conn 2010; Batchelor and Garthwaite 1997; Brasnjo and Otis 2001; Huang et al. 2004; Viaene et al. 2013). (3) mGluR5 displays a preferential perisynaptic localization at the edges of asymmetric synapses formed by both vGluT1 (i.e. cortical) and vGluT2 (brainstem, cerebellum) glutamatergic synapses in contact with thalamocortical cells, suggesting that mGluR5 in the motor thalamus and CM is located to subserve modulatory role over cortical and sub-cortical glutamatergic afferents. Although most vGluT1-positive terminals associated with mGluR5 likely originate from layer VI corticothalamic cells, as suggested for other thalamic nuclei, the sources of vGluT2 terminals may be more heterogeneous and variable between thalamic nuclei. In the VLp, the cerebellar dentate and interposed nuclei likely account for most vGluT2-labeled terminals, while in VApc and CM, additional glutamatergic inputs from the reticular formation, pedunculopontine region and subthalamic nucleus must be considered (Graziano et al. 2008; Rico et al. 2010; Kuramoto et al. 2011; Rovo et al. 2012). Further tracing/mGluR5 immunogold studies combined with optogenetic activation of specific glutamatergic afferents from known vGluT2-positive brainstem neurons are needed to clarify this issue. (4) Our findings demonstrate that pre-synaptic vesicle-filled dendrites of GABAergic interneurons express extrasynaptic and perisynaptic mGluR5 at cortical and sub-cortical glutamatergic synapses. Because GABAergic interneurons are non-existent from the rodent ventral thalamus and caudal intralaminar complex (Jones 2007), these results are indicative of a primate-specific mGluR5-mediated regulation of GABAergic interneurons in these thalamic nuclei. Although the role of group I mGluRs in thalamic function remains poorly characterized, data from the visual thalamus have shed some light on the importance of group I mGluRs in

differentially regulating retinogeniculate glutamatergic synapses on thalamocortical cells vs GABAergic interneurons. While group I mGluRs-mediated slow EPSPs can be evoked in interneurons through activation of retinal afferents, the effects of retinogeniculate synapses on projection neurons rely solely of fast ionotropic transmission (Sherman 2014). Evidence that pre-synaptic group I mGluRs reduce glutamatergic transmission at retinal synapses on thalamocortical cells has also been reported (Govindaiah et al. 2012b). Our findings suggest that the role of mGluR5 in regulating glutamatergic synapses upon GABAergic interneurons in the ventral motor thalamus and CM might differ from what has been proposed in the visual thalamus. Because the VApc and CM do not receive strong "driver-like" afferents similar to retinogeniculate terminals, the effects of mGluR5 on the regulation of interneurons and projection neurons in these nuclei might be restricted to corticothalamic synapses.

Therapeutic relevance of thalamic mGluR5 in Parkinson's disease and L-DOPA-induced Dyskinesia (LID)

Early studies showed that mGluR5 antagonism could ameliorate motor dysfunction in animal models of PD (Conn et al. 2005; Ossowska et al. 2007; Phillips et al. 2006). Negative allosteric modulators (NAMs) that exhibit non-competitive inhibition of mGluR5, such as MPEP (2-methyl-6-(phenylethynyl)-pyridine) and MTEP (3-((2-Methyl-1,3-thiazol-4-yl)ethynyl)pyridine hydrochloride), were effective in relieving motor symptoms and LID in a variety of rodent and nonhuman primate models of PD (Breysse et al. 2002, 2003; Gasparini et al. 2008; Amalric 2015; Coccurello et al. 2004; Ambrosi et al. 2010; Hovelso et al. 2012; Morin et al. 2013; Nicoletti et al. 2015). Further evidence for antidyskinetic efficacy of mGluR5 antagonists in LID come from preclinical studies in rodent (6-OHDA lesion) and monkey (MPTP lesion) PD models (Mela et al. 2007; Levandis et al. 2008; Gasparini et al. 2008; Rascol et al. 2014; Maranis et al. 2012; Sebastianutto and Cenci 2018; Litim et al. 2017; Morin et al. 2016). The mGluR5 negative allosteric modulator (NAM) mavoglurant (AFQ056) was recently tested in PD patients in clinical phase II and III studies (Berg et al. 2011; Schaeffer et al. 2014; Wang et al. 2018). This drug, as well as another mGluR5 NAM, dipraglurant (ADX-48621), has shown potential antidyskinetic action in PD patients, without reducing the efficacy of antiparkinsonian therapy (Rascol et al. 2014; Stocchi et al. 2013). Despite promising pre-clinical and clinical evidence for the potential benefit of mluR5-related drugs in PD and LID, much remains to be known about the mechanisms through which these therapeutic effects are mediated (Battaglia et al. 2004; Masilamoni et al. 2011; Johnston et al. 2010; Breysse et al. 2003; Morin et al. 2014; Berg et al. 2011; Zerbib et al. 2011). Although various hypotheses have been put forward, these were largely focused on mGluR5-mediated regulatory effects of glutamatergic circuits within the BG. The results of the present localization study lay the foundation for a deeper understanding of group I mGluR-mediated physiological effects in the motor thalamus and CM and its potential therapeutic benefits in PD and other brain diseases.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights statement All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Emory University, and were performed according to the Guide for the Care and Use of Laboratory Animals and the U.S. Public Health Service Policy on the Humane Care and Use of Laboratory Animals.

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